Reconstitution, spectroscopy and redox properties of the photosynthetic recombinant cytochrome b_{559} from higher plants.

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Abstract A study of the *in vitro* reconstitution of sugar beet cytochrome b_{559} of the photosystem II is described. Both *α* and *β* cytochrome subunits were first cloned and expressed in *Escherichia coli*. *In vitro* reconstitution of this cytochrome was carried out with partially purified recombinant subunits from inclusion bodies. Reconstitution with commercial heme of both (*αα*) and (*ββ*) homodimers and (*αβ*) heterodimer was possible, the latter being more efficient. The absorption spectra of these reconstituted samples were similar to that of the native heterodimer cytochrome b_{559} form. As shown by electron paramagnetic resonance and potentiometry, most of the reconstituted cytochrome corresponded to a low spin form with a midpoint redox potential +36 mV, similar to that from the native purified cytochrome b_{559}. Furthermore, during the expression of sugar beet and *Synechocystis* sp. PCC 6803 cytochrome b_{559} subunits, part of the protein subunits were incorporated into the host bacterial inner membrane, but only in the case of the *β* subunit from the cyanobacterium the formation of a cytochrome b_{559}-like structure with the bacterial endogenous heme was observed. The reason for that surprising result is unknown. This *in vivo* formed (*ββ)* homodimer cytochrome b_{559}-like structure showed similar absorption and electron paramagnetic resonance spectral properties as the native purified cytochrome b_{559}. A higher midpoint redox potential (+126 mV) was detected in the *in vivo* formed protein compared to the *in vitro* reconstituted form, most likely due to a more hydrophobic environment imposed by the lipid membrane surrounding the heme.

Keywords: Cytochrome b_{559}, electron paramagnetic resonance, reconstitution, redox titration.

Abbreviations

Abs Absorbance

BCA bicinchroninic acid

Cyt cytochrome

β-DM n-dodecyl-*β*-D-maltoside

DEAE diethyl aminoethyl cellulose

E_h ambient redox potencial

E_m midpoint redox potential

EPR electron paramagnetic resonance

ε extinction coefficient

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HP high potencial

HS high spin

IP intermediate potencial

IPTG isopropyl *β*-D-1-thiogalactopyranoside

KDS potassium dodecyl sulphate

LHCP light-harvesting chlorophyll-protein

LP low potential

LS low spin

MBP maltose-binding protein

MES 2-(N-Morpholino)ethanesulfonic acid

OD optical density

PAGE polyacrylamide gel electrophoresis

PMSF phenylmethanesulfonylfluoride

PS photosystem

SDS sodium dodecyl sulphate

TRIS tris(hydroxymethyl)aminomethane

Introduction

Cytochrome b_{559} (Cyt b_{559}) is an integral component of the photosystem II (PSII) reaction center (Stewart and Brudvig 1998). It comprises two small polypeptides, *α* (9 kDa) and *β* (4.5 kDa) subunits, encoded by *psbE* and *psbF* genes, respectively. Each subunit contains one alpha-helix that spans the thylakoid membrane, with the N-terminus in the stromal side (Guskov et al. 2009). The *b*-type heme is coordinated to two histidines in a planar axial structure (Babcock et al. 1985; García-Rubio et al. 2003), one from each subunit, and it is located towards the stromal side (Picorel et al. 1994; Guskov et al. 2009). Despite many attempts, Cyt b_{559} function remains unclear. It has been demonstrated that Cyt b_{559} is essential for the correct assembly of the PSII (Pakrasi et al. 1989; Swiatek et al. 2003), although it is not involved in the primary electron transfer within PSII (Stewart and Brudvig 1998). One of the most accepted hypotheses put forward suggests its involvement in PSII protection against photoinhibition (Stewart and Brudvig 1998; Hung et al. 2010).

Cytochrome b_{559} has singular redox properties among *b*-type cytochromes. It exhibits several midpoint redox potential (E_m) forms (Cramer and Whitmarsh 1977; Ortega et al. 1988; Thompson et al. 1989; Roncel et al. 2001): a high-potential form (HP, $E_m \approx +400$ mV), an intermediate-potential form (IP, $E_m \approx +200$-150 mV), and a low-potential form (LP, $E_m \approx +100$ mV). The HP form is very labile as it has only been observed in intact chloroplasts and some isolated PSII preparations (Stewart and Brudvig 1998). Purified Cyt b_{559} displayed only the LP form (Metz et al. 1983). The molecular mechanisms responsible for these singular redox properties are mainly unknown, although several hypotheses have been proposed (Metz et al. 1983; Babcock et al. 1985; Roncel et al. 2001; Kaminskaya et al. 2007; Shibamoto et al. 2008). The degree of the heme exposure to solvents and its environmental hydrophobicity seem to modulate the Cyt b_{559} redox properties (Ortega et al. 1988; Ahmad et al. 1993; Kaminskaya et al. 1999; Roncel et al. 2001).

Cytochrome b_{559} is a paramagnetic species, thus electron paramagnetic resonance (EPR) spectroscopy is a suitable technique to characterize this metalloprotein. The principal values of the g-tensor are $g_Z \approx 3.05\text{-}2.9$, $g_Y \approx 2.26\text{-}2.15$, and $g_X \approx 1.5\text{-}1.4$, which correspond to a low spin (LS) heme center (Babcock et al. 1985; Stewart and Brudvig 1998; Yruela et al. 2003).

Some *in vitro* and *in vivo* studies have been carried out with the *β* subunit alone. It was found that the *β* subunit was able to undergo (*ββ*) homodimerization and bound the heme group to make a Cyt b_{559}-like structure. It showed spectral and redox properties similar to those of native Cyt b_{559}. *In vitro* reconstitutions with commercial heme and the *β* subunit from the cyanobacterium *Synechocystis* sp. PCC 6803 were described, either from chemically synthesized peptide (Francke et al. 1999) or from the inclusion bodies of *Escherichia (E.) coli* expressing *β* subunit in (Prodohl et al. 2005). Furthermore, *in vivo* formation of the (*ββ*) homodimeric Cyt b_{559}-like structure was reported when the *β* subunit from *Synechocystis* (Prodohl et al. 2005) or *Synechococcus* sp. PCC 7002 (Yu et al. 2003) was expressed in *E. coli*. The polypeptide was integrated into the host bacterial inner membrane as a (*ββ*) homodimer, and then it bound bacterial endogenous heme to make a Cyt b_{559}-like structure.

In the present study, we first report the expression in *E. coli* of the two Cyt b_{559} subunits *α* and *β* from plant species, and the *psbE* gene from a cyanobacterium. With the recombinant subunits from sugar beet (MBP-Rsub*α* and MBP-Rsub*β*), we studied the ability of *in vitro* reconstitution of *αα*, *ββ* and *αβ* forms, the heterodimer being more efficient. We also describe the insertion of *α* and *β* subunits from sugar beet and *Synechocystis* (MBP-Ssub*α* and MBP-Ssub*β*), and the *β* subunit from maize (MBP-Msub*β*) in *E. coli* inner cytoplasmic membrane during their expression. Only the *β* subunit from the cyanobacterium was able to bind heme to form a Cyt b_{559}-like structure *in vivo*. The spectroscopic and redox properties of the obtained forms are described.

Materials and methods

Cloning and expression of the cytochrome b_{559} subunits.

The *psbE* and *psbF* genes from sugar beet and *Synechocystis* and *psbF* gene from maize were cloned and expressed in *E. coli* as fusion proteins using standard procedures. Genes were obtained from genomic DNA by PCR with specific primers, containing a BamHI site forward oligonucleotides and a HindIII site reverse primers. Each gene was cloned into pMAL-c2X expression vector and the sequence of each final construct was confirmed by DNA sequencing. These constructions will give fusion proteins composed of MBP (maltose-binding protein), a protein carrier, and *α* or *β* Cyt b_{559} subunits.

Escherichia coli TB1 competent cells were transformed with the constructs for expression of each fusion protein. A pre-culture was grown overnight from a selected single colony at 37 ºC in LB broth (Miller) medium in the presence of 100 μg ml^{-1} ampicillin. A larger volume of LB broth with ampicillin was inoculated with the pre-culture on the next day and it was grown at 37 ºC until the OD_{600nm} reached 0.6 units. Then 0.5 mM isopropyl *β*-D-1-thiogalactopyranoside (IPTG) was added to the culture to induce the expression of the fusion proteins for 3 h at 37 ºC, with the exception of the MBP-Ssub*α* that was expressed with 1 mM IPTG for 17 h at 18 ºC. After induction, cells were harvested by centrifugation at 10,000*g* for 5 min and frozen at -20 ºC until use. For protein purification, the bacterial pellet was resuspended in 50 mM HEPES, pH 7.5, 10 mM EDTA, and broken by sonication (Ultrasonic Processor XL 2020 Misonix, Farmingale, NY USA) for 45 min (90 s pulses with 60 s intervals) with sample recipient on ice to avoid heating. Cell extracts were clarified by centrifugation at 15,000*g* for 10 min at 4 ºC, obtaining the insoluble material with the inclusion bodies as a pellet. This pellet was saved and the supernatant centrifuged again at 40,000*g* for 45 min at 4 ºC to sediment small cytoplasmic membrane fragments, which were resuspended in the same buffer and analyzed by UV-Vis absorption and EPR spectroscopies, and redox potentiometric titration.

The saved pellet from the first centrifugation containing the inclusion bodies was washed once by centrifugation (10,000*g* for 5 min at 4 ºC) with 50 mM HEPES, pH 7.5, 10 mM EDTA, 1% (w/v) Triton X-100, resuspended with 20 mM TRIS-HCl, pH 8.0, 50 mM sodium dodecyl sulphate (SDS) and saved to make subsequently *in vitro* reconstitutions.

Total protein concentration was determined using the BCA reagent (Pierce Thermo Scientific, Rockford, IL USA). Proteins were separated on SDS-PAGE [12% or 20% (w/v) acrylamide and 4 M urea] and revealed by Coomassie Brilliant Blue staining.

In vitro reconstitution

In vitro heterodimeric Cyt b_{559} reconstitution was done using equimolar amounts of MBP-Rsub*α* and MBP-Rsub*β* fusion proteins obtained as explained above. The detergent SDS of the fusion protein mixture was exchanged with 0.15 mM n-dodecyl *β*-D-maltoside (*β*-DM) by precipitating with 50 mM KCl. After incubating for 10 min on ice, the precipitated potassium dodecyl sulphate (KDS) was removed by centrifugation at 6,000*g* for 15 min at 4 ºC. Finally, equimolar amounts of heme from chemical hemin chloride (Fluka, Buchs, Switzerland) dissolved as described by Kroliczewski and Szczepaniak (2002), were added to the fusion protein mixture. After *in vitro* reconstitution, protease cleavage was performed with Factor Xa to release the Cyt b_{559} subunits from the MBP. Cleavage was done in 20 mM TRIS-HCl, pH 8.0, 1 mM $CaCl_2$, 0.15 mM *β*-DM with 10 units of Factor Xa per mg of fusion protein at 22 ºC for 4 h. The reaction was stopped by adding 1 mM PMSF protease inhibitor. The resultant cleavage mixture was purified by weak anionic-exchange chromatography with a TSK Toyopearl DEAE 650s (TOSOH Bioscience GmbH, Stuttgart, Germany) column pre-equilibrated with 20 mM TRIS-HCl, pH 8.0, and 0.15 mM *β*-DM. After sample loading, the column was washed with five column volumes of the same buffer, and the material eluted with a 0-500 mM NaCl continuous gradient in the same buffer at a flow rate of 0.5 ml min^{-1} in 1-ml fractions. Fractions were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining, and those containing Cyt b_{559} subunits were pooled and

concentrated using a Centriprep or Centricon 3000 NMWL filter (Millipore, Billerica, MA USA). Since some hemes could have been detached during the chromatography, commercial heme was added again in excess to the concentrated sample to ensure maximum reconstitution, and, immediately after, the mixture was passed through three consecutive desalting PD-10 columns (GE Healthcare) to remove most of free heme (Kroliczewski and Szczepaniak 2002). The final sample was concentrated ten times using a Centricon 3000 NMWL filter tube, and the degree of Cyt b_{559} reconstitution was determined by UV-Vis spectroscopy. *In vitro* (*αα*) and (*ββ*) homodimeric Cyt b_{559} reconstitutions were obtained in the same way but using one type of fusion protein only.

Absorption spectroscopy

Visible absorption spectra in the 400-600 nm range were obtained with a Beckman DU 640 spectrophotometer (Beckman Coulter, Brea, CA USA). Samples were measured either in air-oxidized or reduced with 10 mM sodium dithionite. The spectra were compared at the α band at around 559 nm of the reduced *minus* oxidized differential absorption spectra. Two extinction coefficients (*ε*) were used to determine the extent of the cytochrome reconstitution using the difference absorption spectra, *i.e.*, $\varepsilon_{559.5nm\ -\ 577nm}$ = 21.5 mM^{-1} cm^{-1} or $\varepsilon_{559.5nm\ -\ isosbestic\ point}$ = 17.5 mM^{-1} cm^{-1} (Stewart and Brudvig 1998).

Electron paramagnetic resonance

EPR measurements were recorded with a Bruker ESP380E spectrometer (Bruker, Karlsruhe, Germany) working at the X-band. For low-temperature measurements, an Oxford CF935 liquid helium continuous-flow cryostat (Oxford Instruments, Eynsham, UK) was used. Typical conditions for continuous-wave EPR (CW-EPR) measurements were: temperature, 15K; microwave frequency, 9.70 GHz; microwave power, 3.2 x 10^{-2} mW; modulation amplitude, 3.0 Gauss. Two-pulse echo induced EPR (2p ei-EPR) experiments were performed by using the

(π/2 - τ - π - τ) sequence, and detecting the echo intensity as a function of the applied magnetic field. The experimental conditions were 6K and 9.78 GHz.

Some ei-EPR spectra showed a spurious contribution most probably from a Cu(II) species (see Results). In order to make the contributions from heme centers easier to see, a numerical subtraction of this spurious signal was performed. From a sample where the relative intensity of the heme signals was low, the Cu(II) signal in the field interval (270-360 mT) was isolated. This signal was subtracted from the measured spectra for the Cyt b_{559} reconstituted samples. Additionally, a numerical "adjacent averaging" filter was used in order to improve the signal-to-noise ratio without losing the signals resolution.

Potentiometric redox titrations

Potentiometric redox titrations were carried out basically as described by Guerrero et al. (2011). For titrations, samples were suspended in 2.5 ml buffer containing 40 mM MES-NaOH, pH 6.5, 0.587 mM *β*-DM, and the following redox mediators: 10 μM 2,5-dimethyl-*p*-benzoquinone (E'_{m7} = +180 mV), 20 μM *o*-naphtoquinone (E'_{m7} = +145 mV), 2.5 μM N-methyl-phenazonium methosulfate (E'_{m7} = +80 mV) and 20 μM duroquinone (E'_{m7} = +5 mV). Experiments were done at 20 ºC under argon atmosphere and continuous stirring. Reductive titrations were performed by first oxidizing with 25 μM potassium ferricyanide and then reducing it stepwise with small aliquots of 0.1 M sodium dithionite. After addition of sodium dithionite, the absorption spectrum between 500-600 nm range and the redox potential of the solution were simultaneously recorded by using, respectively, a SLM Aminco DW2000 UV-Vis spectrophotometer and a Metrohm potentiometer (Metrohm Ltd., Herisau, Switzerland) provided with a combined Pt-Ag/AgCl microelectrode (Microelectrodes Inc, Bedford, NH USA) previously calibrated against a saturated solution of quinhydrone (E'_{m7} = +280 mV at 20 ºC). Differential spectra of Cyt b_{559} were obtained by subtracting the absolute spectra recorded at each E_h during titrations from the spectra of the fully oxidized cytochrome. The absorbance difference at 559 nm *minus* 570 nm

obtained from these spectra was converted into percentages of reduced cytochrome and plotted *versus* E_h. The E_m values were then determined by fitting the plots to the Nernst equation for one-electron carrier (n= 1) with 1 or 2 components as needed, and using a non-linear curve-fitting program Origin 6.0 (Microcal Software, Piscataway, NJ USA).

Results

Expression of cytochrome b_{559} subunits in *E. coli*

Both Cyt b_{599} subunits, *α* and *β*, of sugar beet were cloned and expressed separately in *E. coli* as fusion proteins with MBP. Due to the marked hydrophobicity of the Cyt b_{559} subunits, MBP was chosen as fusion protein carrier to increase the subunit solubility during expression (Kapust and Waugh 1999). The fusion proteins (MBP-Rsub*α* and MBP-Rsub*β*) corresponded to about 30% of total protein content in cell extracts. Both proteins were expressed both in insoluble and soluble forms (Fig. 1), and a significant part was also incorporated spontaneously into the bacterial cytoplasmic membrane (Fig. 1, lanes 4).

In vitro reconstitution

Inclusion bodies containing the fusion proteins were reconstituted by detergent exchange as explained in Materials and methods, followed by cleavage with Factor Xa to release the *α* and *β* subunits from their own fusion proteins. SDS-PAGE and Coomassie Brilliant Blue staining analysis showed that the cleavage of the fusion proteins was almost complete, as the main band at around 50 kDa almost disappeared (this band corresponds to the fusion protein made of MBP and the corresponding Cyt b_{559} subunit, Fig. 2a). After centrifugation, to eliminate potential aggregates, the resultant components from the proteolytic cleavage were separated by a weak anionic-exchange TSK Toyopearl DEAE 650s chromatography. The washing elution fractions did not show detectable protein content (data not shown), so most of the material remained bound to the column. The fractions eluted at around 140 mM salt contained highly

pure MPB, and the fractions eluted at around 250 mM salt contained the reconstituted Cyt b_{559} (Fig. 2a). These latter fractions were contaminated with other proteins as revealed by SDS-PAGE and Coomassie Brilliant Blue staining. However, the degree of contamination cannot strictly be assessed from the band staining since it is well noticed that the Cyt b_{559} subunits stain very poorly with Coomassie Brilliant Blue stain (Cramer et al. 1986). Thus the Cyt b_{559} subunit concentration in these fractions was much higher than the staining may indicate. Note that the reason to reconstitute the Cyt b_{559} before its purification was to favour the elution of the two subunits coordinated to the heme group with no free heme in solution.

The redox difference absorption spectra of the fractions containing both subunits were very similar to that of native Cyt b_{559} (Fig. 2b), with maxima around 559 and 530 nm, proving the heme remained properly bound to the subunits during the purification process. Indeed, the elution chromatography fractions containing the subunits were actually reddish, indicating the presence of the cytochrome, while MBP fractions were colourless. Despite these interesting data, there was not a close correspondence between the concentration of reconstituted cytochrome, as assessed by the difference absorption spectra, and that of the protein subunits, as determined considering there were two MBP proteins per each *α/β* subunits in the original non-cleaved sample (pure MBP content eluted from the column was determined using an ε_{280nm} = 68,750 $mM^{-1}cm^{-1}$, Bretton and Hofnung 1996). This may indicate that the reconstitution was not complete and/or some hemes were released during the chromatography. To improve the reconstitution, supplementary commercial heme was added to the eluted reconstituted sample, and the absorption spectra were taken again (Fig. 3). The new spectrum was similar to that of native Cyt b_{559}, except the Soret band maximum of the oxidized state shifted from 413 nm (Babcock et al. 1985) to 407 nm. This fact may suggest the presence of some free heme, being more exposed to the environment (Francke et al. 1999). However, the spectral band amplitudes

(Fig. 3) were bigger than that from the original eluted reconstituted samples (Fig. 2b) as expected.

Since *in vitro* studies with Cyt b_{559} *β* subunit have shown that this polypeptide is prone to homodimerize in a (*ββ*) Cyt b_{559}-like structure (Francke et al. 1999; Prodohl et al. 2005), we analyzed the behaviour of the two Cyt b_{559} subunits separately. *In vitro* reconstitution of each subunit alone and the corresponding difference absorption spectra were obtained as above. Comparing the difference spectra of all three types of reconstitutions (*αβ*, *αα* and *ββ*), it was observed that the heterodimer performed somewhat better than the homodimers in terms of spectral shape and band amplitude (Fig. 3). Indeed, *α s*ubunit clearly has less capacity for reconstitution than *β* subunit, and (*αβ*) seems to perform better than the others two (Fig. 3, inset). It is worth mentioning that the heterodimer is the form found in nature.

The *in vitro* reconstituted (*αβ*) heterodimer Cyt b_{559} was analyzed by continuous wave EPR (CW-EPR) and two pulses echo-induced EPR (2p ei-EPR) spectroscopies (Fig. 4). At magnetic field positions corresponding to g_{ef} = 6.0, g_{ef} = 4.3, and g_{ef} = 2.0 CW-EPR showed several features that are not related to the low spin (LS) heme centers (Fig. 4a). The g_{ef} = 4.3 signal is indicative of a non-heme iron and the other two signals (g_{ef} = 6.0 and 2.0) may correspond to a high spin heme (HS) center (at g_{ef}= 2.0 there could also be additional contributions from free radicals). Although native Cyt b_{559} appears essentially as in LS form (Babcock et al. 1985; Stewart and Brudvig 1998; Yruela et al. 2003), some works have reported a low content of HS forms (Fiege et al. 1995; Shuvalov et al. 1995), which could be associated with heme center heterogeneity (Babcock et al. 1985) or heme coordination distortion (Kropacheva et al. 2003).

The CW-EPR spectrum additionally displayed characteristic features corresponding to LS heme (g_{ef} = 2.96, g_{ef} = 2.49, and g_{ef} = 2.28, Fig. 4a). The last signal showed a unique shape at the high field spectral region. Comparing it with the native Cyt b_{559} CW-EPR spectrum, we could suggest a less intense additional signal at g_{ef} = 2.26. These features should be

associated with others at higher magnetic fields, but the weak signals over 350 mT were masked by the baseline contributions of the EPR cavity in our CW-EPR spectrum. As in previous work (Garcia-Rubio et al. 2006), 2p ei-EPR was used to resolve the broad unresolved signals in the high field spectrum region.

The 2p ei-EPR spectrum showed an intense feature at $g_{ef} \approx 2.08$ (not shown), which shape and position indicated that it was probably due to a Cu(II) center. Although it seemed relatively intense in the 2p ei-EPR spectrum, this signal was most probably minor as it is nearly isotropic in comparison with LS heme contributions. A numerical subtraction of this Cu(II) signal was performed in order to better display the heme features (see Materials and methods). After numerical subtraction of this Cu(II) signal the spectrum displayed the LS heme features already detected in CW-EPR, and two additional signals over 350 mT at $g_{ef} = 1.91$ and $g_{ef} = 1.4$ (Fig. 4b).

These EPR results can be understood assuming that two distinct LS heme forms were present in the *in vitro* reconstituted heterodimeric Cyt b_{559} sample. The first LS form (LS1) was similar to the native Cyt b_{559} and corresponded to axial bis-histidine coordination to the heme with characteristic features at $g_z = 2.96$, $g_y = 2.28$, and $g_x = 1.4$ (Babcock et al. 1985; Cramer et al. 1986; Stewart and Brudvig 1998; Yruela et al. 2003). The second form, LS2 with $g_z = 2.49$, $g_y = 2.26$, and $g_x = 1.91$ had also been detected (Blumberg and Peisach 1971; Shuvalov et al. 1995) and was assigned to heme centers with one histidine and one hydroxyl group (-OH) as axial ligands. It is not possible to quantify exactly the LS2 form content in the sample, but according to its lower intensity and smaller g-tensor anisotropy, the relative content of LS2 form would be less than 20%, compared to the LS1 form.

The E_m of the *in vitro* reconstituted heterodimeric Cyt b_{559} was measured by potentiometric reductive titrations at pH 6.5 (Fig. 5). Differential absorption spectra in the spectral *α*-band region were obtained by subtracting the absolute spectrum recorded at +355 mV from those

recorded during the course of the redox titration (Fig. 5a). It was possible to determine the E_m of Cyt b_{559} measuring the relative content of reduced Cyt b_{559} from the absorbance difference between 559 (main peak) and 570 (isosbestic point) nm (Fig. 5a). A plot of the percentages of reduced Cyt b_{559}, obtained from these difference spectra, *versus* E_h could be fit to a Nernst equation for one n= 1 component (Fig. 5b). Only one redox species was found with an E_m = +36 mV, which corresponds to the Cyt b_{559} LP form, the only one present in the purified native Cyt b_{559} from PSII (Matsuda and Butler 1983; Ortega and Hervás 1989).

In vivo formation

A good part of both sugar beet fusion proteins (MBP-Rsub*α* and MBP-Rsub*β*) was spontaneously integrated into the bacterial cytoplasmic membrane, as assessed by SDS-PAGE and Coomassie Brilliant Blue staining analysis (Fig. 1). To test the ability for *in vivo* Cyt b_{559} formation of each plant Cyt b_{559} subunit, redox differential absorption spectra of *E. coli* inner membrane fragments were carried out (Fig. 6). Membranes from non-transformant *E. coli* exhibited redox difference absorption spectra that resembled those of native Cyt b_{559}, and were caused by endogenous *b*-type cytochromes present in the bacterial inner membrane (Gennis 1987). Thus two controls were necessary - induced TB1 cells without transformation and induced TB1 cells bearing empty pMAL-c2X vector. Furthermore, bacterial membrane fragments that expressed *Synechocystis* MBP-Ssub*β* were used as a positive control (Prodohl et al. 2005). As it can be observed, the spectral *α*-band of bacterial inner membrane fragments expressing MBP-Rsub*β* (Fig. 6a) or MBP-Rsub*α* (Fig. 6b) was much smaller than that from membranes fragments with MBP-Ssub*β*, and also somewhat smaller than that from membranes without transformation. This latter result was unexpected and it could be because the expression and/or insertion into the cytoplasmic membrane of the fusion proteins may interfere with the endogenous cytochromes. In any case, the data clearly indicated that the subunits from sugar beet were unable to form a Cyt b_{559}-like structure in the bacterial inner

membrane, but the *β* subunit from *Synechocystis* showed a good formation of such Cyt b_{559}-like structure. The amplitude of the spectral *α*-band observed in membrane fragments containing the MBP-Ssub*β* (Fig. 6a) was around 3.4 times higher than that of MBP-Rsub*β* (Fig. 6a) and 4.7 times higher than that of the MBP-Rsub*α* (Fig. 6b). These differences were already noted when the bacterial cultures were harvested. Indeed, the culture pellets expressing the MBP-Ssub*β* were brownish, indicating a high concentration of cytochromes, but those expressing MBP-Rsub*β* or MBP-Rsub*α* lacked that distinct colour.

The obtained positive results with the MBP-Ssub*β* along with the fact that no studies on *α* subunit from *Synechocystis* has been reported, it prompted us to study the *in vivo* formation of the MBP-Ssub*α*. The redox differential absorption spectrum of membrane fragments containing MBP-Ssub*α* (Fig. 6b) was very similar to that containing the MBP-Rsub*α*, demonstrating that *α* subunit of *Synechocystis* was also unable to perform *in vivo* formation.

Note that the spectra of the *in vivo* formation samples were somewhat distorted in the Soret region causing variable spectral band ratios. This was most probably due to the high light scattering produced by these samples containing large particles of cytoplasmic membranes fragments of different sizes. The presence of the reductant sodium dithionite may also increase that scattering. However, this should not be a significant problem for the purpose of the present work since we always used the spectral *α* band at around 559 nm, where the scattering is much lower than in the Soret region, to compare samples and to calculate de degree of cytochrome formation.

As the protein sequence of the *β* subunit is highly conserved in all organisms analyzed, the negative result with the plant sugar beet was somewhat surprising considering the positive results obtained with *β* subunit from *Synechocystis*. The mRNA encoding *β* subunit from sugar beet and some other plants is modified by RNA-editing, changing a Phe for a Ser at position 26 in the protein sequence (Ser26Phe) (Bock et al. 1993) (all other photosynthetic organisms

already have the Phe codon at that position). This change due to RNA-editing that occurs in certain higher plants is not possible in bacteria (Maier et al. 1996). Thus *β* subunit from sugar beet expressed in bacteria maintained a Ser at position 26 instead of a Phe. To determine if the presence of Ser26 in the recombinant *β* subunit sequence impaired its reconstitution, we expressed the *β* subunit from maize (MBP-Msub*β*) in *E. coli* (note that *psbF* gene from maize already encodes Phe residue at position 26). When the redox differential absorption spectrum of membrane fragments containing the MBP-Msub*β* from maize was compared with that of the *β* subunit from sugar beet, no differences were observed between both samples (data not shown). Thus the Phe26 residue was not essential for *in vivo* formation with the *β* subunit.

Since *β* subunit from *Synechocystis* was the only polypeptide able to form spontaneously a Cyt b_{559}-like structure in the host *E. coli* inner membrane, we further characterized such a protein structure by EPR spectroscopy and potentiometry. To distinguish between the properties of the formed (*ββ*) Cyt b_{559}-like structure from other *b*-type endogenous cytochromes and other potential interferences induced by the expressed protein carrier, MBP, we also studied the bacterial membrane fragments expressing the empty pMAL-c2X vector (negative control).

CW-EPR spectra (Fig. 7a) showed features at g_{ef} = 6.0, g_{ef} = 4.3 and g_{ef} = 2.0 values that were present both in the negative control and in the membrane fragments containing (*ββ*) homodimer. They corresponded to HS heme, non-heminic iron or endogenous radicals located in the *E. coli* inner membrane. Besides, LS heme contributions were also detected both in the negative control and in the membrane fragments containing *(ββ)* homodiner. A part of these signal intensities come from endogenous heme centers located in the *E. coli* inner membrane but the LS heme contribution in the CW-EPR spectrum of the negative control (not shown) was clearly much less intense than the one detected for membrane fragments containing *(ββ)* homodimers. When the CW-spectrum of the negative control was subtracted from that of the

(*ββ*) homodimer sample (Fig. 7a), the remaining features at g_{ef} = 2.96 and g_{ef} = 2.28 should correspond to the (*ββ)* homodimeric Cyt b_{559}–like structure (Fig. 7a).

The ei-EPR spectrum showed again intense features at $g_{ef} \approx 2.08$ (Cu(II) signal) and g_{ef} = 2.0 (signal from endogenous free radicals). These features are truncated in Fig. 7b for a shake of clarity. Besides, the ei-EPR spectrum displayed characteristic features (g_{ef} = 2.96, g_{ef} = 2.28, and g_{ef} = 1.5), typical of a LS heme analogous to that previously reported for native Cyt b_{559} (Babcock et al. 1985; Stewart and Brudvig 1998; Yruela et al. 2003). Thus this sample showed only a LS form due to (*ββ)* homodimeric Cyt b_{559}-like structure within *E. coli* membrane that corresponds to a heme with a planar axial bis-histidine coordination as in the case of native Cyt b_{559} (Babcock et al. 1985; Stewart and Brudvig 1998; Yruela et al. 2003).

Potentiometric reductive titrations were carried out at pH 6.5 (Fig. 8) in the same redox conditions than those for *in vitro* reconstitution. *E. coli* inner membrane fragments containing *β* subunit exhibited two redox species with different E_m (*i.e*., +58 mV and +126 mV) (Fig. 8). Note that the measurements were achieved in *E. coli* membrane fragments, where endogenous cytochromes were also present as mentioned above. As in the case of absorption and EPR measurements, these endogenous hemoproteins should also be detected in the potentiometric measurements. As a control, potentiometric reductive titrations of *E. coli* inner membrane fragments containing MBP only were carried out, and only one E_m of +60 mV was calculated (Fig. 8). These results suggested that E_m = +58 mV form, found in membrane fragments containing *β* subunit was due to bacterial endogenous cytochromes, and the E_m = +126 mV form corresponded to the formation of (*ββ)* homodimeric Cyt b_{559}-like structure. The higher E_m (+126 mV) obtained in the bacterial membranes should not be due to the fact that the *β* polypeptide is forming a fusion protein with the MBP, a soluble moiety. Preliminary experiments indicated that the fusion proteins are anchored within the bacterial cytoplasmic membrane by the alpha-helix of the Cyt b_{559} subunits since protease treatment actually liberated the MBP

moiety, remaining the alpha-helix in the membrane pellet after centrifugation (data not shown). So the soluble moiety of the fusion proteins is very well separated from the cytochrome subunits making it difficult to have any significant influence on the redox properties of *in vivo* formed Cyt b_{559}-like structure.

Discussion

In recent years, *in vitro* reconstitutions of various *b*-type cytochromes have been reported. For instance, recombinant membrane proteins as Cyt b_5 (Mulrooney and Waskell 2000), Cyt b_6 (Kroliczewski and Szczepaniak 2002) and (*ββ*) homodimer Cyt b_{559}–like structure (Prodohl et al. 2005), have been described. In the present study, we report for the first time *in vitro* reconstitution of the photosynthetic Cyt b_{559}, a cytochrome comprised of two different protein subunits that coordinate a heme group. It has to be noted that the spectral *α*-band around 559 nm of the heterodimer appeared somewhat less distorted and with higher amplitude than those from the homodimers, indicating thar (*αβ*) heterodimerization dominates over (*αα*) or (*ββ*) homodimerization. This may explain why nature has selected the heterodimer as the functional form in oxygenic photosynthesis. However, we still cannot discard the formation of some (*αα*) and (*ββ*) homodimers during the *in vitro* (*αβ)* heterodimer reconstitution. We have tried to separate the different potential dimeric forms (*αβ*, *αα*, *ββ*) of the reconstitution mixture by using native PAGE gel with mild conditions and probe with the corresponding antibodies, but all dimeric forms were destroyed during the electrophoresis, the subunits *α* and *β* appearing as separated bands. Such instability of the *in vitro* reconstituted Cyt b_{559} is not totally surprising, considering the 3-D structure of this cytochrome, where the three moieties (*α*, *β*, heme) are highly exposed to the electrophoresis reactants.

Besides the native EPR form LS1, two additional forms, HS and LS2 were found in the reconstituted heterodimeric sample from plants (Fig. 4). Both HS and LS2 forms have also

been found in native Cyt b_{559} (Loew 1983; Scheidt and Gouterman 1983; Shuvalov et al. 1995; Kropacheva et al. 2003) and, according to the features observed in other metalloproteins such as catalase, haemoglobin and myoglobin (Blumberg and Peisach 1971; Fiege et al. 1995), assigned to heme distorted environment (HS) and to heme centers with one of the coordinated histidines displaced by hydroxyl group (LS2). The intensity of the LS2 signal in the native Cyt b_{559} EPR spectra depended on sample integrity and treatments (Fiege et al. 1995; Bianchetti et al. 1998). A model has been suggested in which the two subunits remained linked, but one histidine ligand was replaced by an –OH group as second axial ligand (Shuvalov et al. 1994). It is well possible that some heterodimers are not perfectly assembled *in vitro*, because the protein subunits are free in solution, in contrast to more restricted conditions within the biological membrane. The detergent present in the solution could also add some structural distortion, although small amounts of SDS detergent was proved even to facilitate the *in vitro* reconstitution (Weber et al. 2011).

The E_m (+36 mV) of our *in vitro* reconstituted samples was very similar to that found in isolated native Cyt b_{559} (Matsuda and Butler 1983; Ortega and Hervás 1989), though this redox potential is influenced by the pH and the hydrophobicity surrounding the Cyt b_{559} heme center (Ortega et al. 1988; Ahmad et al. 1993; Kaminskaya et al. 1999; Roncel et al. 2001; Weber et al. 2011).

Expressed foreign integral membrane proteins in *E. coli* have a natural tendency to aggregate into inclusion bodies or insert into the bacterial inner membrane (Drew et al. 2003). Examples of spontaneous insertion into the bacterial membrane are the recombinant LHCP (Kohorn and Auchincloss 1991), Cyt f (Rothstein et al. 1985), Cyt b_5 (Smith et al. 1994) and Cyt b_6 (Kroliczewski et al. 2005). But these three cytochromes were made of a single polypeptide, and were obtained as holoprotein using bacterial endogenous heme. Recent works have also described transformant bacterial membranes with the Cyt b_{559} β subunit from *Synechocystis*

(Prodohl et al. 2005) and *Synechococcus* (Yu et al. 2003), with spectral properties analogous to those of native Cyt b_{559}.

According to our SDS-PAGE analysis, both Cyt b_{559} subunits from the different organisms used in the present study were inserted spontaneously into the bacterial inner membrane (Fig. 1). However, among all Cyt b_{559} subunits analyzed in the present study, *β* subunit from *Synechocystis* was the only polypeptide able to form a Cyt b_{559}-like structure spontaneously in *E. coli* inner membrane. The negative results obtained with *α* subunit from all organisms tested, suggest that there are some constrains for the correct heme assembly independently of protein insertion within the bacterial membrane. This negative result was not due to less stability of the recombinant *α* polypeptide within the bacterial membrane due to, for instance, specific membrane protease degradation since its size remained the same as the native one (Fig. 1). Note that we also detected a poorer *in vitro* reconstitution capacity for this subunit (see Fig. 3).

At present we do not have any clue to explain why overexpressed *β* subunit from plant sugar beet unlike *Synechocystis* was unable to form a Cyt b_{559}-like structure in *E. coli* inner membrane, though its sequence is highly conserved and in both cases the subunits were integrated in the bacterial membrane as full polypeptides. The chloroplastic origin of our plant protein subunits does not seem to account for it since recombinant Cyt b_6 from spinach was successfully reconstituted in bacteria (Kroliczewski et al. 2005).

EPR analysis of bacterial inner membrane fragments containing the *β* subunit from *Synechocystis* showed only a LS1 form, with similar spectroscopic features to those of native Cyt b_{559} (Babcock et al. 1985; Stewart and Brudvig 1998; Yruela et al. 2003). This proves the heme coordination to *in vivo* formed (*ββ*) homodimer. It is worth of noting that LS2 was not detected in these samples, indicating that the formation of a distorted Cyt b_{559} was restricted within the biological membrane most probably due to less hydroxylation activity within the hydrophobic membrane matrix. In addition, the EPR features of this LS1 form shows a small

shifting when compared to those of the LS1 form found in *in vitro* reconstituted Cyt b_{559}. These discrepancies may be connected with some small differences in the environment surrounding the LS heme centers in both cases (Yruela et al. 2003).

Furthermore, previous E_m measured for the (*ββ*) homodimer (+50 mV) were smaller than that obtained in the present work (+126 mV) (Francke et al. 1999; Yu et al. 2003). It has to be noted that these potentiometric measurements were carried out in samples where the (*ββ*) homodimer was outside of a biological membrane and, therefore, close to that of our *in vitro* reconstitution. The observed differences indicate the important role of the environment surrounding the heme on its E_m value (Ortega et al. 1988; Ahmad et al. 1993; Kaminskaya et al. 1999; Roncel et al. 2001). In fact, the heme environment of the *in vivo* formation and the natural thylakoid membrane conditions is more hydrophobic than the aqueous buffer solutions used for *in vitro* reconstitution. Interestingly, the native Cyt b_{559} LP form in PSII membranes exhibits a redox potential value of +110 mV (Roncel et al. 2001), similar to that measured in this work in bacterial inner membrane fragments.

Concluding, we have developed a methodology to reconstitute the *αβ* heterodimeric form of a plant Cyt b_{559}. This can be useful tool to future studies to elucidate the mechanism by which Cyt b_{559} matures, and to explore the molecular mechanisms that regulate the intriguing redox potential variability of this elusive metalloprotein. This methodology can even be more powerful if used together with site-directed mutagenesis techniques, and the application of advanced spectroscopic techniques with no interferences from other tetrapyrrol molecules such as chlorophylls as normally occurred when using photosynthetic materials. Future work will also aim to determine the different behaviour of *α* subunit compared to *β* subunit for *in vivo* formation despite their structural similarity.

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Figures Captions

Fig.1 SDS-PAGE (12%, w/v) in 4 M urea of fractions obtained from the recombinant bacterium *E. coli* TB1 induced with IPTG. **a** TB1 cells expressing MBP-Rsub*α*; **b** TB1 cells expressing MBP-Rsub*β*. MW, molecular weight markers; lane 1, total cellular extract; lane 2, pellet after the first centrifugation containing the inclusion bodies; lane 3, supernatant after the first centrifugation; lane 4, pellet after the ultracentrifugation (*i.e.*, bacterial inner membrane fragments); lane 5, supernatant after the ultracentrifugation; lane 6, total cellular extract of TB1 cells without plasmid (control). The arrows indicate the fusion proteins.

Fig.2 *In vitro* Cyt b_{559} reconstitution with recombinant *α* and *β* subunits of plant sugar beet. **a** SDS-PAGE analysis of the reconstitution: MW, molecular weight markers; lane 1, reconstituted inclusion bodies before cleavage with Factor Xa; lane 2, after cleavage with Factor Xa; lane 3, sample loaded onto the TSK DEAE Toyopearl 650s column after cleavage and centrifugation to eliminate potential aggregates; lane 4, pooled fractions that contain the MBP protein carrier and eluted around 140 mM NaCl; lane 5, pooled fractions that contain reconstituted Cyt b_{559} and eluted around 250 mM NaCl. **b** Redox difference absorption spectrum of the pooled brownish fractions containing the Cyt b_{559} eluted from the DEAE column. The spectrum was obtained in the 500-600 nm spectral range between the reduced and the oxidized samples.

Fig.3 Redox differential absorption spectra of *in vitro* reconstitution of (*αβ*), black, (*αα*), red, and *(ββ)*, blue. The inset shows an enlargement in the 500-600 nm range for a better comparison. All spectra were normalized to the total protein content of reconstituted samples.

Fig.4 EPR measurements of *in vitro* reconstituted (*αβ*) heterodimeric Cyt b_{559}. Panel **a,** CW-EPR spectra. LS features are marked with asterisks, features not related to LS Cyt b_{559} centers are marked with arrows (see text). Panel **b,** 2p ei-EPR (T = 96 ns) spectra (the spurious Cu(II) signal subtracted). The line indicates the signal from non-heme iron at g_{ef} = 4.3, open arrows for LS1 form, solid arrows for LS2 form; g_{ef} values of these signals are indicated.

Fig.5 Potentiometric reductive titration of *in vitro* reconstituted (*αβ*) heterodimeric Cyt b_{559}. **a** Difference absorption spectra in the spectral *α*-band region of the Cyt b_{559}. The spectra were obtained by subtracting absolute spectra recorded during the course of the redox titration *minus* the spectrum recorded at +355 mV. **b** Plot of the percentages of reduced Cyt b_{559} obtained from the absorbance differences at 559–570 nm *versus* ambient redox potentials. Solid curves represent the best fit of the experimental data to the Nernst equation in accordance with one-electron processes (n= 1) for one component.

Fig.6 Redox differential absorption spectra of bacterial TB1 inner cytoplasmic membrane fragments. **a** Induced bacteria without transformation (black); containing the empty pMal-c2X vector (red); expressing MBP-Ssub*β* fusion protein (green); expressing MBP-Rsub*β* fusion protein (blue); the inset shows an enlargement in the 500-600 nm spectral range for a better comparison. **b** Induced bacteria without transformation (black); containing the empty pMal-c2X vector (red); expressing MBP-Rsub*α* fusion protein (green); expressing MBP-Ssub*α* (blue); the inset shows an enlargement in the 500-600 nm spectral range for a better comparison. The spectra were normalized to the total protein content of the corresponding cytoplasmic membrane fragment used.

Fig.7 EPR measurements of (*ββ)* homodimers of *E. coli* cytoplasmic membrane. Panel **a,** CW-EPR spectra. The spectrum was obtained by subtracting the CW-EPR spectrum of cytoplasmic membrane fragments without *(ββ)* homodimers (see text), in order to eliminate the LS contributions from endogenous membrane cytochromes. The signals that do not correspond to LS heme centers are marked with arrows; the LS features are marked with asterisks. Panel **b,** 2p ei-EPR (τ= 96 ns) spectrum of (*ββ*) homodimers of *E. coli* cytoplasmic membrane. The g_{ef} = 2.1-2.0 magnetic field region was truncated in order to get more detail of the relevant LS heme features. The arrows mark the positions of the relevant detected signals; the g_{ef} values of the signals are indicated.

Fig.8 Potentiometric reductive titrations of *E. coli* TB1 inner membrane fragments. Plots of the percentages of reduced Cyt b_{559} obtained from the absorbance differences at 559–570 nm *versus* ambient redox potentials. Solid curves represent the best fits of the experimental data to the Nernst equation in accordance with one-electron processes (n= 1) for one or two components. Cytoplasmic membrane fragments containing the *β* subunit from *Synechocystis* (black); cytoplasmic membrane fragments containing the MBP protein only (red). For other details see caption of Fig. 5 and Materials and methods section.